

FINAL REPORT

Study Title

**BOVINE CORNEAL OPACITY AND PERMEABILITY ASSAY WITH
TWO TIME EXPOSURES AND OPTIONAL HISTOLOGY**

Test Substances

BL, BB, BM,
BP, BQ, BK

Authors

John W. Harbell, Ph.D.
Allison Hilberer, B.A.

Study Completion Date

December 13, 2005

Performing Laboratory

Institute for In Vitro Sciences, Inc.
21 Firstfield Road, Suite 220
Gaithersburg, MD 20878

Study Number

05AE08-AE15.350064

Laboratory Project Number

4140

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STATEMENT OF COMPLIANCE

The Bovine Corneal Opacity And Permeability Assay With Two Time Exposures and Optional Histology of the test substances, BL, BB, BM, BP, BQ, and BK, was conducted in compliance with the U.S. FDA Good Laboratory Practice Regulations as published in 21 CFR 58, the U.S. EPA GLP Standards 40 CFR 160 and 40 CFR 792, and the OECD Principles of Good Laboratory Practice in all material aspects with the following exceptions:

The identity, strength, purity and composition or other characteristics to define the test substances have not been determined by the testing facility.

John W. Harbell, Ph.D.
Study Director

Date

QUALITY ASSURANCE STATEMENT

Study Title: Bovine Corneal Opacity and Permeability Assay with Two Time Exposures and Optional Histology

Study Number: 05AE08-AE15.350064

Study Director: John Harbell, Ph.D.

This study has been divided into a series of in-process phases. Using a random sampling approach, Quality Assurance monitors each of these phases over a series of studies. Procedures, documentation, equipment records, etc., are examined in order to assure that the study is performed in accordance with the U.S. FDA Good Laboratory Practice Regulations (21 CFR 58), the U.S. EPA GLP Standards (40 CFR 792 and 40 CFR 160) and the OECD Principles of Good Laboratory Practice and to assure that the study is conducted according to the protocol and relevant Standard Operating Procedures.

The following are the inspection dates, phases inspected and report dates of QA inspections of this study:

Phase Inspected	Audit Date(s)	Reported to Study Director	Reported to Management
Protocol and Initial Paperwork	18-Jul-05	18-Jul-05	18-Jul-05
Initial Opacity	25-Jul-05 27-Jul-05	26-Jul-05 27-Jul-05	26-Jul-05 28-Jul-05
PAI – Quality Control	29-Aug-05	19-Sep-05	19-Sep-05
Histology – Positive Control and 05AE11, 3 minutes	23-Nov-05	23-Nov-05	05-Dec-05
Final Report and Data	13-Dec-05	13-Dec-05	13-Dec-05

This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

Amanda K. Ulrey, RQAP-GLP
Quality Assurance

Date

SIGNATURE PAGE

**BOVINE CORNEAL OPACITY AND PERMEABILITY ASSAY WITH
TWO TIME EXPOSURES AND OPTIONAL HISTOLOGY**

Initiation Date: July 18, 2005

Completion Date: December 13, 2005

Sponsor:

Sponsor's Representative:

Testing Facility: Institute for In Vitro Sciences, Inc.
21 Firstfield Road, Suite 220
Gaithersburg, MD 20878

Archive Location: Institute for In Vitro Sciences, Inc.
Gaithersburg, MD 20878

Study Director: _____
John W. Harbell, Ph.D. Date

Laboratory Management: Greg Mun, B.A.

Principle Investigator (slide preparation): David A. Hodge HT (ASCP)
Pathology Associates,
A Charles River Company

Histological Evaluation performed by: John W. Harbell, Ph.D.

TEST SUBSTANCE AND REFERENCE SUBSTANCE RECEIPT

IIVS Test Substance Number	Sponsor's Designation	Physical Description	Receipt Date	Storage Conditions*
05AE08	BL	clear colorless non-viscous liquid	7/1/05	room temperature
05AE09	BB	clear colorless non-viscous liquid	7/1/05	room temperature
05AE10	BM	clear colorless non-viscous liquid	7/1/05	room temperature
05AE12	BP	clear colorless non-viscous liquid	7/1/05	room temperature
05AE13	BQ	clear colorless non-viscous liquid	7/1/05	room temperature
05AE14	BK	clear colorless non-viscous liquid	7/1/05	room temperature

* - Protected from exposure to light

**BOVINE CORNEAL OPACITY AND PERMEABILITY ASSAY WITH
TWO TIME EXPOSURES AND OPTIONAL HISTOLOGY**

INTRODUCTION

The Bovine Corneal Opacity and Permeability Assay (BCOP) was used to assess the potential ocular irritancy of the test substances to isolated bovine corneas. Bovine corneas, obtained as a by-product from freshly slaughtered animals, were mounted in special holders and exposed to the test substances. An *in vitro* score was determined for each of two exposure times tested for each test substance based on the induction of opacity and permeability (to fluorescein) in the isolated bovine corneas.

The purpose of this study was to evaluate the potential ocular irritancy of the test substances as measured by changes in opacity and permeability (to fluorescein) in isolated bovine corneas. The laboratory phase of this study was conducted from July 25, 2005 to November 26, 2005 at the Institute for In Vitro Sciences, Inc. Three corneas were treated with each test substance at two exposure times of 3 and 10 minutes. Based on changes in corneal opacity and permeability (relative to the control corneas), an *in vitro* score was determined at each exposure time.

MATERIALS AND METHODS

Bovine Eyes

Bovine eyes were obtained from a local abattoir as a by-product from freshly slaughtered animals (J.W. TREUTH & SONS, Inc., Baltimore, MD). The eyes were excised and then placed in Hanks' Balanced Salt Solution, containing Penicillin/Streptomycin (HBSS), and transported to the laboratory on ice packs. Immediately upon receipt of the eyes into the laboratory, preparation of the corneas was initiated.

Preparation of Corneas

The eyes were grossly examined for damage and those exhibiting defects were discarded. The tissue surrounding the eyeball was carefully pulled away and the cornea was excised such that a 2 to 3 mm rim of sclera was present around the cornea. The isolated corneas were then stored in a petri dish containing HBSS until they were mounted in a corneal holder. The corneas were mounted in the holders with the endothelial side against the O-ring of the posterior chamber. The anterior chamber was then positioned on top of the cornea and the screws were tightened. Starting with the posterior chamber, the two chambers were then filled with Minimum Essential Medium (EMEM) without phenol red, containing 1% fetal bovine serum and 2 mM L-glutamine (Complete MEM). Each corneal holder was uniquely identified with a number written in permanent marker, on both the anterior and posterior chambers. The corneal holders were incubated at $32 \pm 1^\circ\text{C}$ for a minimum of 1 hour.

Controls

The positive control used in this study was neat ethanol (Pharmco). The negative control used in this study was sterile, deionized water (Quality Biological).

Test Substance Preparation

As instructed by the Sponsor, each test substance was administered to the test system without dilution.

Test Substance pH Determination

The pH of each test substance was determined using pH paper (EMD Chemicals Inc./ EM Science). Initially, each test substance was added to 0-14 pH paper with 1.0 pH unit increments to approximate a narrow pH range. Next, each test substance was added to 0-6, 5-10, and/or 7.5-14 pH paper with 0.2-0.5 pH unit increments, to obtain a more accurate pH value. The pH values obtained from the narrower range pH paper are presented in Table 1.

Bovine Corneal Opacity and Permeability Assay

After a minimum of 1 hour of incubation, the corneas were removed from the incubator. The medium was removed from both chambers and replaced with fresh Complete MEM. The initial opacity was determined for each cornea using a Spectro Designs OP-KIT opacitometer. Three corneas, whose initial opacity readings were close to the median opacity for all the

corneas, were selected as the negative control corneas. The treatment of each cornea was identified with the test substance number written in permanent marker on colored tape, affixed to each holder. The medium was then removed from the anterior chamber and replaced with the test substance, positive control, or negative control.

Method for Testing Liquid or Surfactant Materials

The liquid test substances, BL, BB, BM, BP, BQ, and BK were tested neat. An aliquot of 750 μ L of the test substance, positive control, or negative control was introduced into the anterior chamber while slightly rotating the holder to ensure uniform distribution over the cornea. A set of three corneas was incubated in the presence of each test substance at $32 \pm 1^\circ\text{C}$ for 3 minutes. A second set of three corneas was incubated in the presence of each test substance at $32 \pm 1^\circ\text{C}$ for 10 minutes. A set of three corneas was incubated in the presence of the positive control at $32 \pm 1^\circ\text{C}$ for 10 minutes. A set of three corneas was incubated in the presence of the negative control at $32 \pm 1^\circ\text{C}$ for 10 minutes. Each test substance was tested in at least one valid assay. The reference substance, 7948H36-3, was tested in all assays for comparison. After the 3 and 10-minute exposure times, the control or test substance treatments were removed. The epithelial side of the corneas was washed at least three times with Complete MEM (containing phenol red) to ensure total removal of the controls or test substances. The corneas were then given a final rinse with Complete MEM (without phenol red). The anterior chamber was refilled with fresh Complete MEM and an opacity measurement was performed. The corneas were returned to the incubator for approximately 2 hours after which a final measure of opacity was obtained.

After the final opacity measurement was performed, the medium was removed from both chambers of the holder. The posterior chamber was filled with fresh Complete MEM and 1 mL of a 4 mg/mL fluorescein solution was added to the anterior chamber. The corneas were then incubated in a horizontal position (anterior side up) for approximately 90 minutes at $32 \pm 1^\circ\text{C}$. At the end of the 90-minute incubation period, the medium was removed from the posterior chamber and placed into tubes numbered corresponding to chamber number. Aliquots of 360 μ L from the numbered tubes were placed into their designated wells on a 96-well plate. The optical density at 490 nm (OD_{490}) was determined using a Molecular Devices Vmax kinetic microplate reader. If the OD_{490} value of a control or test substance sample was 1.500 or above, a 1:5 dilution of the sample was prepared in Complete MEM (to bring the OD_{490} value within the linear range of the platereader). A 360 μ L sample of each 1:5 dilution was transferred to its specified well on the 96-well plate. The plate was read again and the final reading was saved to a designated print file.

Fixation of Corneas

After the medium was removed for the permeability determination, each cornea was carefully separated from its corneal holder and transferred to an individual prelabeled tissue cassette containing a biopsy sponge. The endothelial surface of each cornea was placed on the sponge to protect it. The cassettes were placed in 10% neutral buffered formalin to fix the corneal tissue for at least 24 hours. The fixed corneas will be stored up to one year.

Histological Evaluation

As instructed by the Sponsor, a histological evaluation was not performed.

Presentation of Data

Opacity Measurement: The change in opacity for each cornea (including the negative control corneas) was calculated by subtracting the initial opacity reading from the final opacity reading. These values were then corrected by subtracting from each the average change in opacity observed for the negative control corneas. The mean opacity value of each treatment group was calculated by averaging the corrected opacity values of each cornea for that treatment condition.

Permeability Measurement: The mean OD₄₉₀ for the blank wells was calculated. The mean blank OD₄₉₀ was then subtracted from the raw OD₄₉₀ of each well (corrected OD₄₉₀). Any dilutions that were made to bring the OD₄₉₀ readings into the linear range of the platereader (OD₄₉₀ should be less than 1.500), had each diluted OD₄₉₀ reading multiplied by the dilution factor. The final corrected OD₄₉₀ of the test substances and the positive control was then calculated by subtracting the average corrected OD₄₉₀ of the negative control corneas from the corrected OD₄₉₀ value of each treated cornea:

$$\text{Final Corrected OD}_{490} = (\text{raw OD}_{490} - \text{mean blank OD}_{490}) - \text{average corrected negative control OD}_{490}$$

The mean OD₄₉₀ value of each treatment group was calculated by averaging the final corrected OD₄₉₀ values of the treated corneas for that treatment condition.

The following formula was used to determine the *in vitro* score:

$$\text{In Vitro Score} = \text{Mean Opacity Value} + (15 \times \text{Mean OD}_{490} \text{ Value})$$

Criteria for Determination of a Valid Test

The BCOP assay was accepted when the positive control (ethanol) caused an *in vitro* score that fell within two standard deviations of the historical mean.

RESULTS AND DISCUSSION

Bovine Corneal Opacity and Permeability Assay

Table 1 summarizes the opacity, permeability, and *in vitro* score for each test substance. Table 2 summarizes the opacity, permeability, and *in vitro* score for the positive control. Since the results of the positive control fell within two standard deviations of the historical mean (within a range of 40.1 to 65.0), the assay was considered valid. The opacity and permeability data for the individual corneas may be found in Appendix B.

Table 1
BCOP Results of the Test Substances

Assay Date	IIVS Test Substance Number	Sponsor's Designation	Conc.	Exposure Time	Mean Opacity Value	Mean OD ₄₉₀ Value	<i>In Vitro</i> Score	pH
7/25/05	05AE08	BL	Neat	3 minutes	-1.0	0.577 ¹	7.7	9.5
				10 minutes	2.7	0.219	6.0	
	05AE09	BB	Neat	3 minutes	0.0	-0.003	0.0	9.0
				10 minutes	2.0	0.000	2.0	
	05AE10	BM	Neat	3 minutes	8.3	0.220	11.6	11.0
				10 minutes	15.7	0.650	25.4	
7/27/05	05AE12	BP	Neat	3 minutes	0.0	0.260	3.9	10.0
				10 minutes	7.7	0.765	19.1	
	05AE13	BQ	Neat	3 minutes	16.7	0.012	16.8	3.5
				10 minutes	30.3	0.215	33.6	
	05AE14	BK	Neat	3 minutes	0.3	0.149	2.6	10.0
				10 minutes	3.0	0.245	6.7	

¹ Cornea #12 showed a much higher OD₄₉₀ than did the other corneas in this treatment group. This led to the higher average OD₄₉₀ for the 3-minute group compared to the 10-minute treatment group (please see values in Appendix B).

Table 2
BCOP Results of the Positive Control

Assay Date	Positive Control	Exposure Time	Mean Opacity Value	Mean OD₄₉₀ Value	<i>In Vitro</i> Score
7/25/05	Ethanol	10 minutes	24.3	1.572	47.9
7/27/05	Ethanol	10 minutes	31.3	1.201	49.3

APPENDIX A

APPENDIX B

BOVINE CORNEAL OPACITY AND PERMEABILITY ASSAY

OPACITY SCORE

<u>TA #</u>	<u>CORNEA #</u>	<u>INITIAL</u>	<u>FINAL</u>	<u>CHANGE</u>	<u>CORRECTED</u>	<u>AVG</u>	<u>STDEV</u>
05AE08	10	5	4	-1	-1.0		
Neat	11	4	3	-1	-1.0		
3 minutes	12	5	4	-1	-1.0	-1.0	0.0
05AE08	14	2	3	1	1.0		
Neat	16	5	5	0	0.0		
10 minutes	17	4	11	7	7.0	2.7	3.8
05AE09	18	5	5	0	0.0		
Neat	20	6	5	0	0.0		
3 minutes	23	5	5	0	0.0	0.0	0.0
05AE09	25	4	5	1	1.0		
Neat	26	6	5	0	0.0		
10 minutes	28	5	10	5	5.0	2.0	2.6
05AE10	29	5	11	6	6.0		
Neat	30	4	16	12	12.0		
3 minutes	31	5	12	7	7.0	8.3	3.2
05AE10	32	5	21	16	16.0		
Neat	36	4	22	18	18.0		
10 minutes	37	4	17	13	13.0	15.7	2.5
Neg. Control	1	4	4	0	NA		
Sterile, DI water	4	4	4	0	NA		
10 minutes	5	3	3	0	NA	0.0	
Pos. Control	6	3	27	24	24.0		
Ethanol	7	6	33	27	27.0		
10 minutes	8	4	26	22	22.0	24.3	2.5

Initial corneal opacity average: 4

NA - Not Applicable

PERMEABILITY SCORE

**Neg. Control
Sterile, DI water
10 minutes**

Cornea #	OD490
1	0.005
4	0.010
5	0.002

Avg.	0.006

**Pos. Control
Ethanol
10 minutes**

Cornea #	OD490	Dilution Factor	Corrected OD490
6	1.025	1	1.019
7	0.461	5	2.299
8	1.403	1	1.397
-----			Avg. = 1.572
			STDEV = 0.658

**05AE08
Neat
3 minutes**

Cornea #	OD490	Dilution Factor	Corrected OD490
10	0.271	1	0.265
11	0.188	1	0.182
12	1.290	1	1.284
-----			Avg. = 0.577
			STDEV = 0.614

**05AE08
Neat
10 minutes**

Cornea #	OD490	Dilution Factor	Corrected OD490
14	0.300	1	0.294
16	0.217	1	0.211
17	0.157	1	0.151
-----			Avg. = 0.219
			STDEV = 0.072

**05AE09
Neat
3 minutes**

Cornea #	OD490	Dilution Factor	Corrected OD490
18	0.001	1	-0.005
20	0.005	1	-0.001
23	0.003	1	-0.003
-----			Avg. = -0.003
			STDEV = 0.002

**05AE09
Neat
10 minutes**

Cornea #	OD490	Dilution Factor	Corrected OD490
25	0.002	1	-0.004
26	0.004	1	-0.002
28	0.010	1	0.004
-----			Avg. = 0.000
			STDEV = 0.004

**05AE10
Neat
3 minutes**

Cornea #	OD490	Dilution Factor	Corrected OD490
29	0.234	1	0.228
30	0.257	1	0.251
31	0.186	1	0.180
-----			Avg. = 0.220
			STDEV = 0.036

**05AE10
Neat
10 minutes**

Cornea #	OD490	Dilution Factor	Corrected OD490
32	0.590	1	0.584
36	0.307	1	0.301
37	1.071	1	1.065
-----			Avg. = 0.650
			STDEV = 0.386

IN VITRO SCORE

In Vitro Score = Mean Opacity Value + (15 x Mean OD490)

Test Article	Concentration	Exposure Period	Mean Opacity	Mean OD490	In vitro Score
05AE08	Neat	3 minutes	-1.0	0.577	7.7
05AE08	Neat	10 minutes	2.7	0.219	6.0
05AE09	Neat	3 minutes	0.0	-0.003	0.0
05AE09	Neat	10 minutes	2.0	0.000	2.0
05AE10	Neat	3 minutes	8.3	0.220	11.6
05AE10	Neat	10 minutes	15.7	0.650	25.4
Ethanol	Neat	10 minutes	24.3	1.572	47.9

BOVINE CORNEAL OPACITY AND PERMEABILITY ASSAY

OPACITY SCORE

<u>TA #</u>	<u>CORNEA #</u>	<u>INITIAL</u>	<u>FINAL</u>	<u>CHANGE</u>	<u>CORRECTED</u>	<u>AVG</u>	<u>STDEV</u>
05AE12	7	4	4	0	0.0		
Neat	8	3	4	1	1.0		
3 minutes	9	4	3	-1	-1.0	0.0	1.0
05AE12	11	4	11	7	7.0		
Neat	12	5	13	8	8.0		
10 minutes	13	3	11	8	8.0	7.7	0.6
05AE13	15	3	18	15	15.0		
Neat	16	3	21	18	18.0		
3 minutes	19	3	20	17	17.0	16.7	1.5
05AE13	20	3	35	32	32.0		
Neat	22	3	34	31	31.0		
10 minutes	23	3	31	28	28.0	30.3	2.1
05AE14	25	4	5	1	1.0		
Neat	26	4	4	0	0.0		
3 minutes	28	3	3	0	0.0	0.3	0.6
05AE14	29	3	5	2	2.0		
Neat	30	5	11	6	6.0		
10 minutes	31	4	5	1	1.0	3.0	2.6
Neg. Control	1	3	3	0	NA		
Sterile, DI water	2	5	5	0	NA		
10 minutes	3	3	3	0	NA	0.0	
Pos. Control	4	4	36	32	32.0		
Ethanol	5	4	37	33	33.0		
10 minutes	6	2	31	29	29.0	31.3	2.1
	*17	5					
	*27	3					
	*41	6					
	*42	6					
	*43	5					
	*44	5					
	*45	5					
	*46	3					
	*47	5					
	*48	5					
	*49	5					
	*50	5					
	*51	6					
	*52	3					

Initial corneal opacity average: 4

* - Corneas not used in this assay, but used to find initial opacity average.
 NA - Not Applicable

PERMEABILITY SCORE

**Neg. Control
Sterile, DI water
10 minutes**

Cornea #	OD490
1	0.005
2	0.009
3	-0.002
Avg.	0.004

**Pos. Control
Ethanol
10 minutes**

Cornea #	OD490	Dilution Factor	Corrected OD490
4	1.225	1	1.221
5	0.962	1	0.958
6	1.428	1	1.424
Avg. =			1.201
STDEV =			0.234

**05AE12
Neat
3 minutes**

Cornea #	OD490	Dilution Factor	Corrected OD490
7	0.287	1	0.283
8	0.282	1	0.278
9	0.224	1	0.220
Avg. =			0.260
STDEV =			0.035

**05AE12
Neat
10 minutes**

Cornea #	OD490	Dilution Factor	Corrected OD490
11	0.852	1	0.848
12	0.869	1	0.865
13	0.587	1	0.583
Avg. =			0.765
STDEV =			0.158

**05AE13
Neat
3 minutes**

Cornea #	OD490	Dilution Factor	Corrected OD490
15	0.014	1	0.010
16	0.002	1	-0.002
19	0.032	1	0.028
Avg. =			0.012
STDEV =			0.015

**05AE13
Neat
10 minutes**

Cornea #	OD490	Dilution Factor	Corrected OD490
20	0.114	1	0.110
22	0.324	1	0.320
23	0.218	1	0.214
Avg. =			0.215
STDEV =			0.105

**05AE14
Neat
3 minutes**

Cornea #	OD490	Dilution Factor	Corrected OD490
25	0.137	1	0.133
26	0.201	1	0.197
28	0.121	1	0.117
Avg. =			0.149
STDEV =			0.042

**05AE14
Neat
10 minutes**

Cornea #	OD490	Dilution Factor	Corrected OD490
29	0.418	1	0.414
30	0.182	1	0.178
31	0.147	1	0.143
Avg. =			0.245
STDEV =			0.147

IN VITRO SCORE

In Vitro Score = Mean Opacity Value + (15 x Mean OD490)

Test Article	Concentration	Exposure Period	Mean Opacity	Mean OD490	In vitro Score
05AE12	Neat	3 minutes	0.0	0.260	3.9
05AE12	Neat	10 minutes	7.7	0.765	19.1
05AE13	Neat	3 minutes	16.7	0.012	16.8
05AE13	Neat	10 minutes	30.3	0.215	33.6
05AE14	Neat	3 minutes	0.3	0.149	2.6
05AE14	Neat	10 minutes	3.0	0.245	6.7
Ethanol	Neat	10 minutes	31.3	1.201	49.3